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Ammonia Intoxication in Rats: Protection by *N*-Carbamoyl-L-Glutamate Plus L-Arginine

(hyperammonemia/mitochondrial carbamoyl phosphate synthetase/urea biosynthesis/acylaminoacid acylase)

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ABSTRACT **Rats given a lethal dose ($LD_{99.9}$) of ammonium acetate (10.8 mmol/kg of body weight) were protected to the extent of 85 and 76% when previously injected with *N*-carbamoyl glutamate or L-arginine, respectively, at a level of 4 mmol/kg of body weight. At a dose of 1 mmol/kg of body weight, L-arginine protected 24%, while *N*-carbamoyl-L-glutamate protected 61% of the animals. When a combination of *N*-carbamoyl-L-glutamate plus L-arginine (1 mmol each per kg of body weight) was injected, *100% of the rats were protected.* The efficacy of *N*-carbamoyl-L-glutamate is related to its role as an activator of mitochondrial carbamoyl phosphate synthetase (EC 2.7.2.5) and its resistance to hydrolysis by tissue acylaminoacid acylase. *N*-Acetyl-L-glutamate, the naturally occurring and most effective activator of mitochondrial carbamoyl phosphate synthetase, was relatively ineffective in protection against lethal dose of ammonium acetate, because of its ready hydrolysis by acylaminoacid acylase.**

The findings reported provide a rational basis for the use of *N*-carbamoyl-L-glutamate plus L-arginine in the prevention and treatment of hyperammonemia in clinical conditions of liver disease and parental infusion of amino acids, and in feeding of urea supplements to ruminants.

Ammonia is a relatively toxic metabolite in ureotelic terrestrial mammals. Ureotelism, in fact, serves to protect such animals from ammonia intoxication by the operation of a highly efficient metabolic pathway for conversion of ammonia to urea. The susceptibility to ammonia intoxication varies somewhat between mammalian species. Rats appear to be particularly sensitive.

An abbreviated representation of the pathway involved in urea biosynthesis in ureotelic animals is shown in Fig. 1. It should be noted that the biosynthetic pathway is initiated in mitochondria by the activation of carbamoyl phosphate synthetase (EC 2.7.2.5) by *N*-acetyl-L-glutamate (reaction **2**). The presence of an enzyme system that catalyzes the synthesis of *N*-acetyl-L-glutamate from acetylcoenzyme A plus glutamate, and which is specifically stimulated by arginine, has been recently described by Shigesada and Tatibana (1, 2) (reaction **1**). The mitochondrial enzyme ornithine carbamoyltransferase (EC 2.1.3.3) catalyzes a reaction of carbamoyl phosphate with ornithine to form citrulline (reaction **3**). *These three enzymes, which are involved in the initial stages of urea biosynthesis, are restricted to liver mitochrondia.* The enzymes that convert the ureido groups of citrulline to urea with the regeneration of ornithine, while most active in liver cytosol, are also present in other tissues (3, 4). Thus, it is clear that urea biosynthesis in liver from ammonia and carbon dioxide is initiated in mitochondria, and that the mitochrondrial phase requires in addition to the enzymes carbamoyl phosphate synthetase and ornithine carbamoyltransferase an effective concentration of ATP, acetylglutamate [or an analogue (5, 6)] to activate carbamoyl phosphate synthetase, and a sufficient concentration of ornithine to insure maximal utilization of carbamoyl phosphate to form citrulline.

The relative instability of carbamoyl phosphate (7, 8) necessitates its rapid conversion to citrulline. This rapid conversion is normally insured by an adequate level of ornithine and the relatively high level of ornithine carbamoyltransferase in liver mitochondria.

While *N*-acetyl-L-glutamate has been shown to be the naturally occurring activator of carbamoyl phosphate synthetase (9), and to be the most effective of a series of compounds tested (4, 5), other closely related analogues (e.g., *N*-carbamoyl-L-glutamate, 2-acetoxyglutarate) are also able to activate the enzyme, but with different K_m values (6).

Greenstein and coworkers (10–14) demonstrated that certain amino acids, including essential amino acids, were highly toxic when injected intraperitoneally into rats. The toxicity was demonstrated to be due to ammonia intoxication resulting from rapid deamination of the amino acids. When arginine (or citrulline or ornithine) was added to an individual amino acid

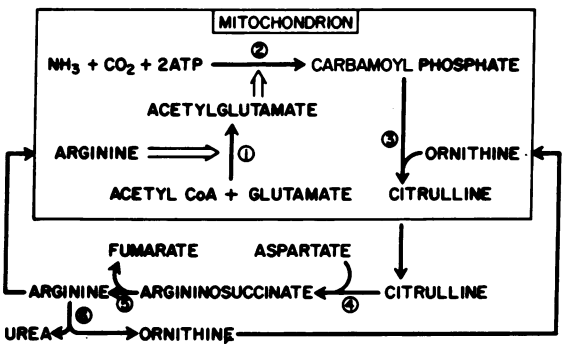

FIG. 1. Enzymatic steps in urea biosynthesis: (*1*) acetylglutamate synthetase; (*2*) carbamoyl phosphate synthetase; (*3*) ornithine carbamoyltransferase; (*4*) argininosuccinate synthetase; (*5*) argininosuccinase; and (*6*) arginase. *Double arrows* indicate activation of (*1*) acetylglutamate synthetase by arginine and (*2*) carbamoyl phosphate synthetase by acetylglutamate.

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or a mixture of amino acids capable of producing ammonia intoxication, significant protection against toxicity was observed. The beneficial effect of arginine (and citrulline or ornithine) was demonstrated to be the result of a more efficient operation of the urea biosynthetic pathway, primarily because of the availability of a high level of ornithine, not only with respect to protection against toxicity of natural amino acids but also against a lethal dose of ammonium acetate. The levels of the enzymes carbamoyl phosphate synthetase, ornithine carbamoyltransferase, and arginase (EC 3.5.3.1), as determined by *in vitro* assay, were shown by Kim (15) not to be changed significantly in livers of rats exposed to ammonia intoxication with or without arginine supplementation.

In an attempt to determine whether factors involved in the operation of the urea cycle other than ornithine (or compounds capable of rapidly forming ornithine) would be effective in protection against ammonia intoxication, Kim (15) studied the effect of *N*-acetyl-L-glutamate and *N*-carbamoyl-L-glutamate. The former proved to have relatively little beneficial effect, even though it is the naturally occurring and most effective activator of carbamoyl phosphate synthetase, while the latter was strikingly effective. At doses of 1 mmol/kg, carbamoylglutamate was even more effective than arginine. Kim (15) also investigated the metabolic fate of [^{14}C]carbamoyl-labeled carbamoylglutamate in rats. About 65% of the carbamoylglutamate injected was excreted unchanged in the urine within 6 hr after intraperitoneal injection, and a total of 75% was accounted for in the urine after 48 hr. No radioactivity was observed after 48 hr in extracts of liver, spleen, kidney, intestine, muscle, and brain. The remaining 25% was presumably catabolized.

Because of the limited number of animals used in the earlier studies, we reinvestigated the effect of carbamoylglutamate with a larger number of experimental animals.

MATERIALS AND METHODS

Materials. Albino Holtzman strain male rats, weighing between 180 and 240 g, were obtained from Holtzman Rat Co., Madison, Wis.; Sprague–Dawley rats were from Carworth Farms, Rockland Co., N.J. The animals were fed a complete ration of Rockland Purina rat chow, and were starved for 24 hr (with water *ad libitum*) before injections. L-Arginine, *N*-acetyl-L-glutamate, and *N*-carbamoyl-L-glutamate were obtained from Sigma Chemical Co.; *N*-carbamoyl-L-glutamate was recrystallized from water before use. Hog kidney acylase I (hydrolyzes 1175 μmol of *N*-acetyl-L-methionine/hr per mg of enzyme) was purchased from Sigma Chemical Co.

Experimental Procedures. Rats, starved for 24 hr, were weighed just before the experiment. Solutions were prepared such that a total of 2.5 ml of solution was administered to each rat. Ammonium acetate, at a dose of 10.8 mmol/kg of body weight [which is the $LD_{99.9}$ for rats (10)] was used throughout these experiments. The compounds to be tested for their protective effect were injected intraperitoneally; 1 hr later, a solution containing a lethal dose of ammonium acetate was injected (10–14). Untreated animals generally died within 30 min after injection of the ammonium acetate solution. Survival of treated animals beyond 30 min after the injection of the ammonium acetate solution was considered to represent protection by the compound injected 1 hr before the ammonium acetate solution.

Assay for Hog-Kidney Acylase I. The reaction mixture contained 0.5 ml of 0.1 M phosphate buffer (pH 7.0); 0.5 ml of 0.1 M substrate (*N*-acetyl-L-glutamate or *N*-carbamoyl-L-glutamate, adjusted to pH 7.0 with NaOH); and 0.5 ml of acylase I preparation. Incubation was at 37° for 10 min. The reaction was stopped by heating the tubes at 100° for 3 min. After centrifugation, an aliquot of the supernatant solution was reacted with ninhydrin solution according to the method of Moore and Stein (16). A blank was prepared by use of boiled acylase I (3 min at 100°) in place of fresh enzyme in the reaction mixture.

RESULTS

Effect of L-arginine and various glutamate derivatives on detoxification of ammonia

Table 1 lists the results obtained on the effect of arginine and a few glutamate derivatives on the survival of rats injected with an $LD_{99.9}$ dose of ammonium acetate. It is evident that 4 mmol of L-arginine and *N*-carbamoyl-L-glutamate protected 85 and 76% of the rats, respectively. However, a dose of 1 mmol of *N*-carbamoyl-L-glutamate per kg is significantly more effective than the same dose of L-arginine. It is also seen that neither *N*-acetyl-L-glutamate nor L-glutamate is effective in protecting rats from a toxic dose of ammonium acetate. It should be noted further that, under these experimental conditions, neither L-arginine nor *N*-carbamoyl-L-glutamate alone afforded complete protection.

Effect of the combined administration of compounds

When 1 mmol/kg each of *N*-carbamoyl-L-glutamate and L-arginine were mixed, and the mixture was administered, *100% of the animals were protected* (Table 2). Combinations of various amounts of these compounds below 1 mmol/kg do not appear to be as effective as the 1 mmol/kg dosage.

Hydrolysis of *N*-acetyl-L-glutamate and *N*-carbamoyl-L-glutamate by kidney acylate

N-Carbamoyl-L-glutamate has a less favorable K_m than *N*-acetyl-L-glutamate as an activator of carbamoyl phosphate synthetase (6). The greater efficacy of carbamoylglutamate in protecting against ammonia intoxication thus must be related to the relative ease of hydrolysis of acetylglutamate by tissue acylases. Acylamino acids are readily hydrolyzed by rat-tissue acylaminoacid acylases, which are present in relatively high amounts in kidney, liver, and brain (17). Evidence for the ready hydrolysis of *N*-acetyl-L-glutamate by hog-kidney acylase I is presented in Table 3. It should be noted that *N*-carbamoyl-L-glutamate is not hydrolyzed at all, even at an

TABLE 1. *Effect of various compounds injected before an $LD_{99.9}$ dose of ammonium acetate*

Compound	Dose (mmol/kg of body weight)	No. of animals	No. of animals survived	Survived (%)
L-Arginine	4	26	22	85
	1	21	5	24
N-Carbamoyl-L-glutamate	4	38	29	76
	1	23	14	61
N-Acetyl-L-glutamate	4	32	7	22
L-Glutamate	4	13	1	8

TABLE 2. *Effect of the combined administration of N-carbamoyl-L-glutamate plus L-arginine on ammonium acetate toxicity*

Mixture*	No. of animals	No. of animals survived	Survived (%)
0.5 mmol L-arginine + 0.5 mmol *N*-carbamoyl-L-glutamate	24	7	29
0.5 mmol L-arginnine + 1.0 mmol *N*-carbamoyl-L-glutamate	16	9	56
1.0 mmol L-arginine + 0.5 mmol *N*-carbamoyl-L-glutamate	26	23	89
1.0 mmol L-arginine + 1.0 mmol *N*-carbamoyl-L-glutamate	30	30	100

* Followed by an $LD_{99.9}$ dose of ammonium acetate.

enzyme concentration 25 times that used with *N*-acetyl-L-glutamate.

While other possible explanations for the greater efficacy of carbamoylglutamate might exist, such as rates of absorption from the peritoneal cavity, transport within the liver cell to the mitochondria, etc., it would seem that the ease of hydrolysis by tissue acylaminoacid acylases of acetylglutamate is the most important factor in explaining the lesser efficacy of acetylglutamate injected into the intact animal.

DISCUSSION

A rationale for the efficacy of the combination of carbamoylglutamate plus arginine in protecting against a toxic dose of ammonium acetate is provided in Fig. 1. Because carbamoyl phosphate synthetase can function only if activated by its naturally occurring activator (*N*-acetyl-L-glutamate), or by an analogue (6, 18), the initiation of ammonia conversion to urea is critically dependent on this step. The amount of *N*-acetyl-L-glutamate present in rat-liver mitochondria has recently been determined (2). They found a concentration of 0.1–0.2 mM, which is of the same order as the K_a value found (18) for the purified carbamoyl phosphate synthetase (0.11 mM). The concentration of acetylglutamate about doubled on a high protein diet, in parallel with increased levels of carbamoyl phosphate synthetase and urea biosynthesis (2). Apparently, the biosynthetic capacity of the acetylglutamate synthetase system is limited, and the level of acetylglutamate maintained in mitochondria is just barely sufficient to maintain an adequate level of activated carbamoyl phosphate synthetase. While arginine is capable of increasing the maximal velocity or acetylglutamate synthetase about 6-fold, and to stimulate the synthesis of acetylglutamate in mitochondria as much as 10-fold (1), it is not known whether the concentration of arginine needed for this stimulation exists in mitochondria. Shigesada and Tatibana (1) do point out, however, that the salutary effect of arginine in protecting against ammonia intoxication may possibly be functioning through this mechanism.

The striking effectiveness of *N*-carbamoyl-L-glutamate in protecting against ammonia intoxication can only be interpreted on the basis of its demonstrated capacity to activate carbamoyl phosphate synthetase (5, 6). As has been pointed out, Kim found that 75% of this compound was excreted unchanged in the urine 48 hr after injection, 65% of it appearing in the first 4 hr (15). Thus, it is not likely that this compound is being converted to some other metabolite. When carbamoylglutamate was fed to rats at low levels (1.9 g/kg of ration), the rats had an increased capacity for citrulline synthesis by their liver mitochondria, as compared to rats fed a glutamate-supplemented diet (19).

It is possible that the beneficial effect of the combination of carbamoylglutamate plus arginine is largely the result of their effect on carbamoyl phosphate synthetase activity, carbamoylglutamate by direct action and arginine by stimulation of the acetylglutamate synthetase system. However, early experiments of Greenstein (10–14) showed that the salutary effect of arginine could be duplicated by ornithine and citrulline, a finding that can only mean that arginine, in addition to its possible role as a stimulator of acetylglutamate synthetase, must be serving as a source of intramitochondrial ornithine. Because of the relatively high level of activity of ornithine carbamoyltransferase as compared to carbamoyl phosphate synthetase in mitochondia (20), the concentration of ornithine normally present in mitochondria may be too low to insure saturation. Elevation of the intramitochondrial concentration of ornithine as a result of arginine feeding could thus be expected to provide more favorable conditions for rapid conversion of low levels of carbamoyl phosphate. While the mechanism of liver ornithine carbamoyltransferase is ordered, with carbamoyl phosphate binding first and ornithine second (8), the rate of the reaction will of course not be optimal if the ornithine level is not high enough to insure saturation.

Chiosa *et al.* (21) reported that *N*-acetylglutamate, *N*-acetylaspartate, *N*-carbamoylglutamate, and *N*-carbamoylaspartate all provided "significant protection" at levels of 5–6 mmol/kg in mice given an LD_{70-90} dose of NH_4Cl. The data as reported, however, showed inconsistencies between groups. The effect of the combination of *N*-carbamoylglutamate plus arginine was not reported.

Hyperammonemia is associated clinically with serious disturbances in cerebral function (22, 23). In adults, the condition is most frequently the result of hepatic failure; the term hepatic encephalopathy has been used to describe this condition .(23). While therapy in these cases has been directed toward lowering the sources of ammonia (low protein intake, reduction of intestinal sources of ammonia by use of antibiotics and other agents, etc) (24, 25), direct therapy has involved the use of arginine or a combination of arginine and glutamic acid (22, 26, 27). The rationale for the use of arginine stems from the studies of Greenstein and coworkers discussed earlier, and that for glutamic acid is based on its potential as a substrate for glutamine synthesis in extrahepatic tissues

TABLE 3. *Effect of hog-kidney acylase I on N-acetyl-L-glutamate and N-carbamoyl-L-glutamate*

Substrate used	Amount of acylase I used (mg)	μmol of substrate hydrolyzed/hr per mg of enzyme
N-Acetyl-L-glutamate	0.02	182
N-Carbamoyl-L-glutamate	0.50	0

(10–14, 27). However, clinical experience with these agents has demonstrated that they are not consistently effective (22).

While repair of a damaged liver would seem to indicate the need for a diet high, rather than low, in protein, clinical experience has demonstrated that a high-protein diet is contraindicated because of the complication of hyperammonia. However, reinvestigation of this problem would seem to be indicated if an efficacious agent were available to prevent hyperammonemia. The use of *N*-carbamoyl-L-glutamate plus L-arginine for this purpose is suggested.

Infusion of amino acids to infants (28, 29) [as well as to adults (30, 31)] is also frequently associated with hyperammonemia. The potential benefit of parental feeding of amino acids to malnourished infants and adults thus must be balanced against the potential hazard of hyperammonemia.

In conditions of hyperammonemia of infants associated with genetic disorders involving enzymes of the ornithine–urea cycle, the capacity for urea biosynthesis appears to be impaired in most instances because of a failure of the ornithine urea cycle to operate efficiently in the face of higher than minimal levels of ammonia (32). Thus, therapy is directed to lowering ammonia intake by means of a low-protein diet and by lowering of intestinal sources of ammonia. The possibility of enhancing the efficiency of the urea biosynthetic pathway by increasing the level of *activated* carbamoyl phosphate synthetase (via carbamoyl glutamate) *plus* an increase in ornithine (via arginine) has not been reported.

An area of possible value for an agent that can protect against ammonia intoxication is that of urea feeding of ruminants. While the purpose of dietary feeding of urea supplements is to provide a cheap nitrogen precursor of protein by exploitation of the capacity of rumen microorganisms to convert urea to ammonia, and in turn to utilize the ammonia for protein synthesis, a complication is occasionally experienced in the way of serious hyperammonemia of the host animal (33). While the use of arginine plus carbamoyl glutamate would not be expected to influence the rumen conversion of urea nitrogen to protein nitrogen, it could serve to protect the host animal against ammonia intoxication by increasing the rate of conversion of absorbed ammonia to urea.

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